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Prediction of Photoperiodic Regulators from Quantitative Gene Circuit Models

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SUMMARY

Photoperiod sensors allow physiological adaptation to the changing seasons. The prevalent hypothesis is that day length perception is mediated through coupling of an endogenous rhythm with an external light signal. Sufficient molecular data are available to test this quantitatively in plants, though not yet in mammals. In *Arabidopsis*, the clock-regulated genes *CONSTANS (CO)* and *FLAVIN, KELCH, F-BOX* (*FKF1*) and their light-sensitive proteins are thought to form an external coincidence sensor. Here, we model the integration of light and timing information by *CO*, its target gene *FLOWERING LOCUS T (FT)*, and the circadian clock. Among other predictions, our models show that FKF1 activates *FT*. We demonstrate experimentally that this effect is independent of the known activation of *CO* by FKF1, thus we locate a major, novel controller of photoperiodism. External coincidence is part of a complex photoperiod sensor: modeling makes this complexity explicit and may thus contribute to crop improvement.

INTRODUCTION

Many eukaryotes measure changes in day length (photoperiod), in order to synchronize their life strategies with seasonal rhythms. The photoperiod sensor in vertebrates is thought to be located in the *pars tuberalis* of the pituitary gland, though its molecular mechanisms are unclear (reviewed in Hazlerigg and Loudon, 2008). Day-length perception in plants occurs in leaves, giving rise to a long-range signal. In *Arabidopsis thaliana*, a signal induced by long photoperiods controls the transition to flowering at the apical meristem. Other plant species initiate overwintering adaptations, such as bud dormancy and tuber formation, in response to short photoperiods (Thomas and Vince-Prue, 1997). Photoperiod measurement depends upon an interaction between photoreceptors and the 24 hr circadian clock. In *Arabidopsis*, the clock-controlled transcription of the B-box factor *CONSTANS* (*CO*) leads to a *CO* mRNA profile that peaks late in the day. High *CO* mRNA levels coincide largely with the light interval under long-day conditions (such as 16L:8D, a cycle of 16 hr of light and 8 hr of darkness), but are restricted to the dark phase under short days (8L:16D) (Suarez-Lopez et al., 2001). The major target of *CO*, the gene *FLOWERING LOCUS T* (*FT*), is expressed after the *CO* peak, but only when *CO* expression coincides with light (Yanovsky and Kay, 2002). This led to the hypothesis that CO may activate *FT* transcription in a light-dependent manner (reviewed in Carré et al., 2006; Imaizumi and Kay, 2006). More recently, the CO protein was shown to be unstable in darkness, partly due to interaction with COP1 (Jang et al., 2008), but to accumulate under constant white or blue light (Valverde et al., 2004). Thus, stabilization of the CO protein in the light may account for the light-dependency of CO effects on *FT*. This regulation occurs in the phloem companion cells (An et al., 2004), allowing rapid transport of the FT protein product to the apical meristem (reviewed in Kobayashi and Weigel, 2007; Turck et al., 2008). There, interaction with the meristem-specific transcription factor FD activates the homeotic genes that lead to floral development (reviewed in Kobayashi and Weigel, 2007; Turck et al., 2008).

This molecular mechanism is consistent with the long-standing hypothesis that day-length perception is mediated through coincidence of an endogenous rhythm with an external light signal (Bünning, 1936). Expressing this hypothesis in equations shows that the rhythmic component could be a generic, clock-controlled gene with expression levels that rise toward the end of a long photoperiod (Oosterom et al., 2004). This contrasts with the "internal coincidence model" that may apply in vertebrates (Hazlerigg and Loudon, 2008) in which photoperiod acts to bring two circadian rhythms into a particular phase relationship (Pittendrigh, 1960). Recent evidence suggests that the mechanism of day-length perception in plants may be more complex than either conceptual model. For example, expression of the *CO* mRNA at the end of long-day photoperiods is mediated in part through the action of a rhythmically

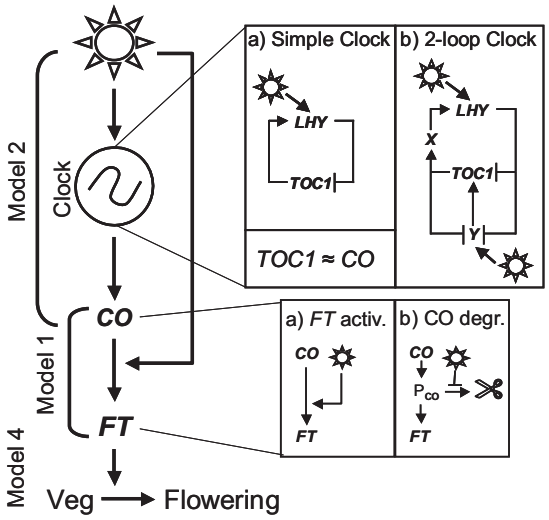

Figure 1. Overview of Modeling Stages

Model 1 uses *CO* mRNA data and light/dark cycles as inputs and simulates *FT* mRNA accumulation. In model 1a, light and *CO* mRNA activate *FT* transcription whereas in model 1b light inhibits the degradation of CO protein, which activates *FT*. Model 2 takes a light/dark cycle as input and simulates the rhythmic expression of *CO* mRNA. Model 2a is based on a single-loop model for the circadian clock (Locke et al., 2005a) while model 2b uses the interlocking-loop model (Locke et al., 2005b). Models 1 and 2 are combined in model 3, which simulates *FT* mRNA profiles for a given light/dark cycle. Speculative models including *FKF1* are presented in Figures 4, S4, S5, and S8. Model 4 uses model 3 to predict flowering responses based on *FT* mRNA accumulation.

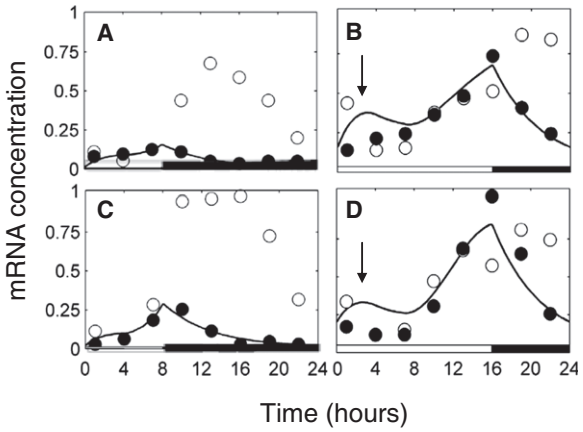

Figure 2. External Coincidence of *CO* and Light Predicts *FT* mRNA Expression

FT mRNA expression patterns were simulated using model 1a, based on the training data sets (A and B) (Yanovsky and Kay, 2002) and (C and D) (Imaizumi et al., 2003), under short-day (A and C) and long-day (B and D) conditions. The maximal *CO* mRNA level under short days is set to 1 for each set of data and simulations. Open circles, *CO* mRNA data; closed circles, *FT* mRNA data; solid line, simulated *FT* mRNA levels. Filled bar on time axis, dark interval; open bar, light interval.

expressed, light-activated F-box-Kelch protein known as FKF1 (reviewed in Imaizumi and Kay, 2006). High FKF1 protein levels coincide with the light interval at the end of a long day, when FKF1-mediated degradation of transcriptional repressors in the CYCLING DOF FACTOR family promote transcription of *CO* (Fornara et al., 2009; Imaizumi et al., 2005; Sawa et al., 2007). Under short day conditions, FKF1 is expressed in the dark and appears inactive. This external coincidence between light and the *FKF1* expression rhythm affects the *CO* expression rhythm: the level of CO protein may therefore integrate the output of two external coincidence sensors.

Here, we model the photoperiod sensor of *Arabidopsis* in detail, based upon molecular timeseries data. We aim to test whether the expression patterns of the known flowering-time genes are quantitatively consistent with their proposed regulatory functions, and whether these functions are sufficient to explain the observed behavior of the plant. Analysis of the models confirms our understanding of flowering time regulation in some areas. Specific failures of the models in other areas predict new regulatory interactions or components that can be tested by molecular experimentation.

RESULTS

Model Construction and Data Selection

The regulatory network was represented in ordinary differential equations, where the form of the equations reflected the known molecular interactions. Model construction proceeded in stages (Figure 1). Alternative models were compared at each stage. The maximal transcription rates, mRNA degradation rates and other biochemical parameters were estimated by fitting the models to quantitative, molecular timeseries data (see the Supplemental Data, available online), as none of these parameter values have been measured experimentally. The consistency of the available data sets enabled this approach. There was little data for key proteins in wild-type plants, so our initial models were based on quantitative mRNA expression patterns, with indirect information on CO protein levels and their regulation by light. Twenty-four sets of timeseries data (Table S1) were selected to construct and validate the models (see Supplemental Data).

Model 1: Activation of *FT* by *CO* and Light

Model 1 aimed to simulate the accumulation of *FT* mRNA, starting from *CO* mRNA expression data. The detailed mechanism of *FT* activation by CO protein remains to be determined, so several models were tested (Figure S1, Supplemental Data). In the simplest model (1a), we assumed that the CO protein was produced rapidly and was highly unstable, so that accumulation of the CO protein mirrored *CO* mRNA. Furthermore we supposed that the CO protein was only active in the light. Thus the rate of *FT* transcription was determined by the level of *CO* mRNA when light was present and *FT* was not transcribed in darkness. Parameter values for this model were estimated using data on *CO* and *FT* mRNA levels in wild-type plants grown under long and short photoperiods (sets 1, 3, 8, and 9, see Table S1), quantified from two publications of the Kay laboratory (Imaizumi et al., 2003; Yanovsky and Kay, 2002). We term these the training data (Figure 2). As expected, a limited number of parameter values allowed accurate simulation of the observed pattern of *FT*

mRNA accumulation (see Supplemental Data). Using the optimal parameter sets, the fit of simulated *FT* mRNA levels to either training data set was better than the fit of the experimental data sets to each other, indicating that no better match to the training data was possible.

A more complex model (1b) explicitly included an unstable CO protein that was stabilized during the light interval but rapidly degraded in darkness. Light-driven accumulation of this protein promoted *FT* transcription. Model 1b also fitted well to the training data, with no significant improvement over model 1a (unpublished data). More complex models, involving for example an additional effect of light on the ability of the CO protein to activate *FT* transcription, failed to improve the fit (see Supplemental Data). Models 1a and 1b were validated using further sets of *CO* and *FT* mRNA data from a variety of photoperiodic conditions. The parameters developed for the training data also fitted the validation data well (Figure S1C; Supplemental Data), indicating that the simple mechanisms of submodels 1a and 1b recapitulated the overall activation of *FT* by *CO*. As model 1b explicitly includes regulation of the CO protein, we anticipate that this will be more useful for comparison to future molecular data.

Modification of *FT* Activation

The quantitative models allowed us to test whether *FT* activation was altered in mutant backgrounds. The *toc1* mutation, for example, shortens the period of the circadian clock from 24 hr to 21 hr. *toc1* mutant plants are induced to flower rapidly under 8L:16D, but this defect in photoperiodism can be rescued by growing the mutants under 21 hr light-dark cycles. It was therefore proposed that the altered circadian clock function is the only photoperiod-response defect in the *toc1* mutant (Yanovsky and Kay, 2002). From the *CO* mRNA levels observed in *toc1* mutants, models 1a and 1b simulated levels of *FT* mRNA that only slightly underestimated the levels observed in *toc1* mutants (Figures S2a–S2d and unpublished data). The best match was obtained by increasing the *FT* activation parameter by 40% (Figures S2e–S2h).

Using model 1a, simulations of *FT* transcription under long days consistently predicted an aberrant peak of *FT* mRNA in the early morning that was absent from the training data (Figures 2B and 2D). The aberrant peak was also predicted using model 1b but was delayed by the time required for CO protein to accumulate (unpublished data, similar to Figure 3D). This defect suggested that our models overlooked an additional aspect of *FT* regulation. The effect of *CO* on *FT* transcription may be "gated," such that accumulation of *CO* mRNA in the morning results in less transcription of *FT* than an equal amount of *CO* mRNA in the afternoon. We estimated the effect of the hypothetical "morning gate" in model 1a and found that it was photoperiod-dependent but modest (~60% reduction in FT activation; see Supplemental Data, Figure S3). However, the RNA data available have insufficient time resolution to constrain the effect accurately, and including the morning gate made only a small contribution to the overall fit under standard long- and short-day conditions. For these pragmatic reasons and considering additional experimental evidence (see Discussion), no separate "morning gate" mechanism was included in subsequent models.

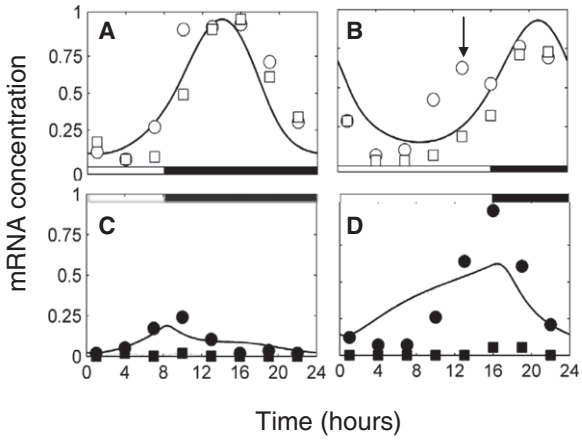

Figure 3. Model 3 Recapitulates *CO* mRNA Profiles of *fkf1* Mutant Plants but Predicts *FT* mRNA Close to Wild-Type

Expression patterns of *CO* mRNA (A and B) and *FT* mRNA (C and D) were simulated using model 3 (solid lines), under short-day (A and C) and long-day (B and D) conditions. The arrow in (B) marks the *FKF1*-dependent shoulder in *CO* expression, which is absent in the model. Expression level data (sets 8 and 9, Table S1, Imaizumi et al., 2003) are shown, as in Figure 2. Open symbols, *CO* mRNA data; filled symbols, *FT* mRNA data; circles, data from wild-type; squares, data from *fkf1* mutant. Filled bar on time axis, dark interval; open bar, light interval.

Model 2: Circadian Regulation of *CO* Transcription

The waveform of *CO* mRNA accumulation is thought to be a crucial component of the photoperiod sensor. To include rhythmic *CO* regulation in model 2, we assumed that *CO* was similar to the clock component *TOC1*, which is expressed at the same phase (Locke et al., 2006; Locke et al., 2005b). We therefore simulated *CO* expression based on the *TOC1* component of existing models for the circadian clock. Two clock models were tested. The simplest, in Model 2a, comprised a single transcriptional feedback loop and a single mechanism of light input at dawn (Figure 1) (Locke et al., 2005a). The entrained phase of this clock model is locked to dawn and the remainder of the photoperiod has no effect (Locke et al., 2006). This model fitted *CO* RNA data poorly, because it could not accommodate the observed change in the *CO* waveform between short and long photoperiods (Figure 2). In contrast, the circadian clock in Model 2b comprised two interlocking feedback loops and was entrained through light inputs to two genes (Figure 1) (Locke et al., 2005b). The interlocking-loop clock model is capable of adjusting its phase relative to dawn in response to varying photoperiods (Locke et al., 2006), resulting in a better fit to *CO* mRNA expression (Figures 3A and 3B).

Model 2b failed to predict the shoulder of *CO* mRNA accumulation that is observed at the end of the light interval under long photoperiod cycles in wild-type plants (Figure 3B, arrow). The simulated *CO* waveforms were closer to data from *fkf1* mutant plants. Since *FKF1* is known to affect *CO* mRNA accumulation at the end of a long day (Imaizumi et al., 2005, 2003; Sawa et al., 2007), the absence of *FKF1* in our model might account for this discrepancy. Under short days, where there is little difference between *CO* waveforms in wild-type and *fkf1* mutants, the model fitted both well. A preliminary model 3F1 was developed

to simulate the effect of *FKF1* on *CO* transcription, using data on the FKF1 protein profile to control additional synthesis of *CO* mRNA in a light-dependent manner (see Supplemental Data, Figure S4). The model fitted one to two time points in the shoulder of *CO* mRNA data, which had a limited effect on *FT* mRNA accumulation. Simulating an *fkf1* mutation in preliminary model 3F1 caused only a 35% reduction in *FT* transcription rate at the end of a long photoperiod. Activation of *CO* transcription by FKF1 in our model represents the double-negative mechanism, in which the CYCLING DOF FACTOR (CDF) repressor proteins are degraded by FKF1, but allows a much simpler mathematical formulation. When further quantified data become available, it should be possible to model recently-discovered details of the molecular mechanisms involved (Fornara et al., 2009; Sawa et al., 2007).

Model 3: Photoperiodic Regulation of *FT*

In order to simulate the regulation of *FT* under the control of light and the circadian clock, submodel 1b was combined with submodel 2b to form model 3 (Figure 1; see Supplemental Data). This model replaced the experimental *CO* mRNA data used in Figure 2 with the simulated *CO* mRNA waveforms shown in Figures 3A and 3B. Importantly, the model parameters were not altered, because each submodel had already been constrained to the relevant data.

The *FT* expression patterns predicted by model 3 remained consistent with the *FT* data from the training and validation data sets (Figures 3C, 3D, and S1d). The *CO* mRNA profile simulated by model 2b lacked the *FKF1*-dependent shoulder at the end of the long photoperiod (Figure 3B), whereas model 1b had matched the *FT* mRNA based on data that included this shoulder. We therefore expected that using the simulated *CO* mRNA profile in model 3 would yield lower levels of simulated *FT* mRNA, compared to model 1b. A *CO* mRNA profile similar to an *fkf1* mutant might simply have yielded the low levels of *FT* mRNA that had been observed in *fkf1* mutant plants. The peak level of simulated *FT* mRNA in model 3 was indeed lower than observed in wild-type plants but, surprisingly, the reduction was only 40% (Figure 3D), which was an overestimate of an order of magnitude compared to the *FT* RNA levels observed in the *fkf1* mutant. Thus model 3 simulated a *CO* mRNA profile similar to the *fkf1* mutant but greatly overestimated the *FT* mRNA level. Removing *FKF1* in the mutant plant caused a much more severe reduction of *FT* mRNA levels than could be predicted from the effect of the *fkf1* mutation on *CO* mRNA levels alone. Consistent with this, adding the FKF1-dependent shoulder to the *CO* mRNA profile in preliminary model 3F1 had predicted only a modest increase in *FT* mRNA levels (Figure S4). Together, these results indicated that a major effect of *FKF1* may be to activate *FT* expression downstream or independently of *CO* mRNA.

To estimate the importance of *FKF1*-dependent activation for *FT* transcription, we constructed a speculative model (model 3F2), in which the observed FKF1 protein profile and the simulated CO protein together promoted *FT* transcription (see supplementary text). The model was matched to the training data sets (8 and 9; see Table S1) that allowed direct comparison of wild-type and *fkf1* mutant results (Figure 4). The new *CO* mRNA profiles were matched to the data for wild-type and *fkf1* mutant plants under long photoperiods, including the *FKF1*-dependent shoulder in the wild-type (Figures 4C and 4E). The model predicted a smaller effect of *FKF1* on *CO* RNA under short photoperiods, consistent with the data (Figure 4B). Simulated *FT* mRNA levels showed an improved profile in wild-type (Figure 4E). The aberrant morning peak of earlier models (Figures 2B and 2D) was removed, because FKF1 levels are low at dawn (Figure S4a). Simulations with model 3F2 showed that 90% of wild-type *FT* transcription at the end of a long photoperiod was FKF1-dependent.

The photoperiod-dependence of this effect was similar to the effect of FKF1 on *CO* transcription. This raised the possibility that CO and FKF1 cooperate to regulate both *CO* and *FT* transcription by a single mechanism, which would include a positive feedback of CO protein upon *CO* mRNA abundance (Figure 4J). To test this hypothesis, the FKF1-dependent shoulder of *CO* mRNA under long photoperiods was measured in wild-type plants and in seven mutant lines carrying *co* alleles that severely affect flowering time (Figure S7). Control *fkf1* mutant plants showed low *CO* mRNA levels 13h after dawn (about 25% of wild-type levels), consistent with Figure 4. *CO* mRNA was undetectable in one insertional mutant *co* allele. The other six alleles had *CO* mRNA levels very close to wild-type, showing that CO protein function was not required for the FKF1-dependent shoulder in *CO* transcription. These data favor the model depicted in Figure 4A, in which FKF1 has two distinct effects: its known regulation of *CO* mRNA levels, which does not require CO protein, and a previously-undescribed effect on *FT* expression, which depends on CO.

We next explored the qualitative patterns of *FT* regulation in response to a range of different light-dark cycles. As the FKF1 protein profiles required for model 3F2 were available for only two conditions, these simulations used model 3. *FT* mRNA levels increased in a non-linear fashion when the system was stably entrained to 24 hr light-dark cycles with longer photoperiods (Figure 5A). Treating a short-day-entrained system with one longer photoperiod had a more graded effect (Figure S5), indicating that circadian entrainment significantly affected the photoperiod response (see Discussion). Data to inform the component models were only available for a limited range of conditions, so it was not unexpected that the clock model did not entrain stably to some exotic light-dark cycles. The model remained strongly rhythmic in these conditions but phase variations between successive cycles indicated that it was not following a stable, entrained limit cycle, which was the condition imposed for our analysis (see Supplemental Data). Nonetheless, two patterns of *FT* regulation appeared physiologically relevant (Figure 5A). First, light-dark cycles of longer or shorter duration than 24 hr partially activated *FT* under all photoperiods and, second, the steepest increase of *FT* expression with photoperiod occurred under 24 hr cycles. Thus the model predicts that there will be an optimal cycle duration to obtain the strongest photoperiodic switch, likely in a 24 hr environment.

Model 4: Prediction of Flowering Time

Published flowering time data differ widely among *Arabidopsis* accessions and across laboratories, reflecting the many

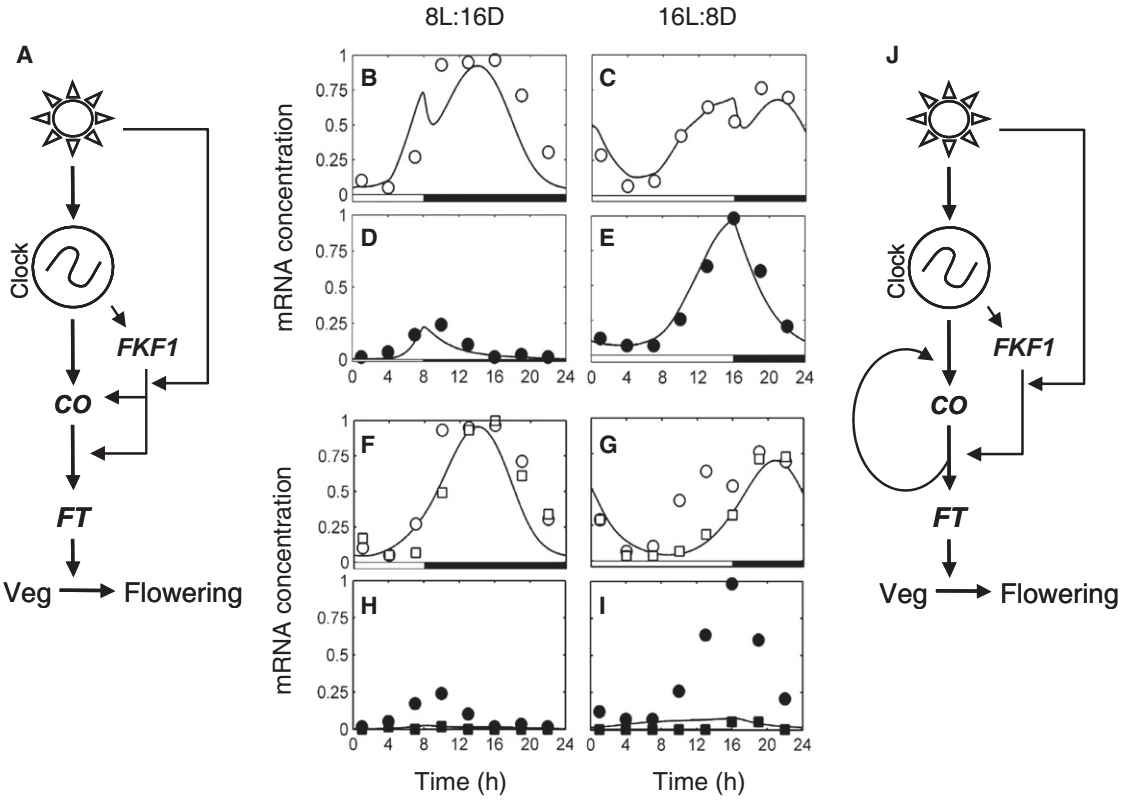

Figure 4. *FKF1* Affects *FT* Activation

Speculative model 3F2 (A) includes light-dependent activation of both *CO* and *FT* by *FKF1*. Simulations (solid lines) can closely match the expression patterns (Imaizumi et al., 2003) of *CO* (B, C, F, and G) and *FT* (D, E, H, and I) mRNA in both the wild-type (B–E) and *fkf1* mutant (F–I) under short-day (B, D, F, and H) and long-day (C, E, G, and I) conditions. An alternative, more parsimonious hypothesis is shown in (J): *FKF1* has a single effect on CO protein activity, which both controls *FT* transcription and feeds back positively to regulate *CO* transcription (see Figure S7). Open symbols, *CO* mRNA data; filled symbols, *FT* mRNA data; circles, data from wild-type; squares, data from *fkf1* mutant. Filled bar on time axis, dark interval; open bar, light interval.

environmental inputs that control absolute flowering time (Boss et al., 2004). Most molecular studies, moreover, have focused on one standard long and short photoperiod condition, so the non-linear relationship between the *FT* mRNA profile and flowering time could not be estimated from the data available (see supplementary text). We therefore compared flowering time data to the *FT* expression profiles predicted by model 3, where we could perform simulations for any photoperiod. Simple mathematical functions fitted well to flowering data for plants of the Columbia accession (Corbesier et al., 1996), with clear differences in the functions required for different experimental protocols (seeSupplemental Data, and Figure S6). A sigmoid function matched data sets from three laboratories that used similar experimental protocols: data on days to flowering in plants of the Columbia accession (Figure S6a, Corbesier et al., 1996) and data on total leaf numbers for plants of the Landsberg(*erecta*) accession (Figure 5B, Wilczek et al., 2009) and the Wassilewskija accession (Figure 5C, Pouteau et al., 2008). The critical photoperiod that elicited the half-maximal flowering response was almost identical in Figures 5B and 5C, despite the differences in absolute leaf numbers (see supplementary information). Mutation of *CO* prevents *FT* expression in the models, so *co* mutant plants are predicted to flower under all photoperiods with the same, high leaf number as wild-type plants under very short photoperiods, as confirmed in the recent data (Wilczek et al., 2009). The flowering function offers a standard approach to reveal robust behavior of the photoperiod response system.

DISCUSSION

The external coincidence model can clearly provide a workable photoperiod sensor (Carré et al., 2006). We aimed for a more detailed, quantitative explanation of the observed molecular regulation that could be linked to the whole-organism response. Our approach will be validated if the resulting models accurately predict the molecular data yet remain comprehensible, if the models direct future experimentation to address gaps in current data, and if the models give insight into comparable processes in other contexts. The data required need not present technical challenges. Consistent data sets for *CO* and *FT* mRNA levels and flowering times would already be valuable to explore a wider range of environmental conditions and genotypes with the full range of *FT* profiles, from *ft* mutants to *FT*-overexpressing lines. These data would test the simple functions assumed here for CO

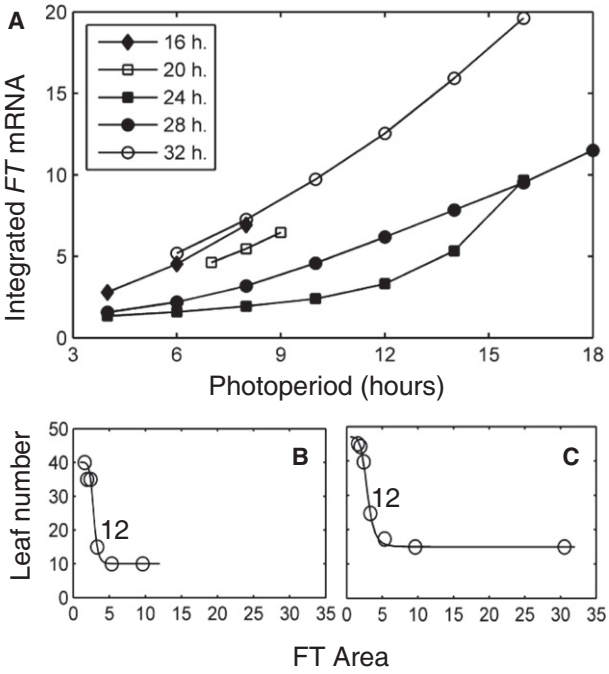

Figure 5. A Photoperiod Sensor for 24 hr Days, Regulating Flowering

(A) *FT* expression was simulated under light-dark cycles comprising 6–16 hr photoperiod in a total cycle duration of 16 hr (diamonds) to 32 hr (open circles), using model 3. 24 hr cycles show the largest ratio of integrated *FT* mRNA between long and short photoperiods. Absent results are due to unstable circadian entrainment in some conditions. (B and C) The integrated *FT* mRNA area simulated by model 3 under different photoperiods (diamonds) is related to flowering time data for the same photoperiods by a simple function (line), with specific parameter values (see Supplemental Data) for data sets from Landsberg(*erecta*) (B, Wilczek et al., 2009) and Wassilewskija (C, Pouteau et al., 2008). Flowering time data covered photoperiods from 6L:18D to 16L:8D in (B) and (C), with an additional data point for constant light in (C). The data point for 12L:12D is labeled (12).

protein synthesis and for the effectiveness of FT as a floral inducer. Current data were sufficient to make eight specific, testable predictions (Table S2), which are discussed below, together with their functional implications for photoperiod responses.

Suppressed Induction of *FT* in the Morning

A good overall fit after parameter optimization shows that a model is consistent with the molecular data. The simplest models of *FT* activation by light and *CO* were largely sufficient to recapitulate the molecular data. Simulating data from the *toc1* mutant confirmed that this mutation in the circadian clock affected *FT* regulation largely by altering rhythmic *CO* mRNA expression (Niwa et al., 2007; Yanovsky and Kay, 2002), though a small (~40%) increase in *FT* activation by another mechanism remains possible (prediction 2, Table S2). A specific failure of the model can be more informative. For example, the overestimation of *FT* levels at the start of a long photoperiod (Figure 2) suggested that another level of regulation reduces the effectiveness of *CO* at this time (prediction 1 in Supplemental Data). A similar, morning-specific suppression has been identified in experimental studies of *CO*-overexpressing plants (Valverde et al., 2004) and linked to phyB in *cop1* mutant plants (Jang et al., 2008). Our models show that this mechanism operates in wild-type plants, quantify its effects, show that the effect is greater in long than in short photoperiods (Figure S3), and suggest a molecular mechanism (Figure 4; see below). The quantitative effect of the suppression was modest (~60% reduction in *FT* activation in Figure S3) and its parameters were not well constrained, because it only affected one to two data points in the *FT* profile. Moreover, morning expression of *FT* can sometimes be observed experimentally (Corbesier et al., 2007), and flowering can be accelerated under exotic light cycles that drive high *CO* expression in the morning (Roden et al., 2002). Thus the morning suppression mechanism is not always effective at the level of *FT* expression and is not always relevant at the level of flowering time, though it is apparent in most of the molecular data sets considered here. Regulation by *FKF1* provides a parsimonious mechanism for the suppressive effect, as detailed below.

Regulation of *CO* by a Photoperiod-Responsive Clock

The circadian peak of *CO* RNA accumulation moves to a later phase under long photoperiods (Figure 2). The interlocking, dual feed-back loop model of the clock was required to match this phase delay (Figure 3, prediction 3 in supplemental table), because the phase of this clock model responds strongly to light at dusk (Locke et al., 2006). A more complex, three-loop clock model (Locke et al., 2006) matches better to data for circadian-regulated genes other than *CO*, where peak phase is much less delayed under long photoperiods (unpublished data; Millar and Kay, 1996). The *CO* entrainment profile is consistent with the two-loop model and therefore might reflect a distinct circadian clock mechanism that is restricted to specific cell types, for example in the vasculature (An et al., 2004). Circadian clocks with distinct entrainment patterns have also been proposed in the photoperiod sensor of the short-day plant, *Ipomoea nil* (Hayama et al., 2007). The unknown mechanism that regulates some aspects of the *CO* waveform independently of the *GI* rhythm (Fornara et al., 2009) might also be consistent with the unusual photoperiod-sensitivity of the timing of peak *CO* RNA.

The dusk-sensitive entrainment of *CO* makes the system more responsive to lengthening photoperiods, particularly to delays in lights-off, than it is to stable, long photoperiods. The sensitivity to photoperiod change arises because the first lengthened photoperiod simply allows a longer duration of light to coincide with a *CO* mRNA rhythm that is set to the early phase characteristic of a short day, as proposed by Bünning's external coincidence mechanism (Bünning, 1936). Under stable, long photoperiods, however, the observed phase of the *CO* rhythm is delayed, moving more of the *CO* mRNA peak into the dark interval. After entrainment to 8 hr photoperiods, for example, model 3 predicted a significantly greater *FT* area on the first 10 hr or 12 hr photoperiod than it did when stably entrained to 10 hr or 12 hr photoperiods (Figure S5). The circadian entrainment of *CO* is consistent with observed flowering responses to changes in the time of sunset, which have evolved exquisite sensitivity in tropical trees (Borchert et al., 2005).

Regulation of *CO* and *FT* by *FKF1* and Photoperiod

Whereas circadian entrainment delays *CO* expression under longer photoperiods, *FKF1* promotes earlier *CO* mRNA accumulation (reviewed in Imaizumi and Kay, 2006). The *FKF1*-dependent shoulder in *CO* RNA accumulation was readily simulated by adding a second source of *CO* RNA to supplement the waveform driven only by the clock model (Figures 3, 4, and S4). This source could be either an arbitrary square waveform (data not shown) or the observed FKF1 protein profile, which also has a sharp onset and decline (Imaizumi et al., 2003). In either case, the sharp change in the additional *CO* was important to match the dip in *CO* RNA profile that is often observed at the end of a long photoperiod (prediction 5).

Our models (Figures 3 and 4) highlighted a dramatic and previously unsuspected effect of *FKF1,* to promote *FT* expression independently of its effect on *CO* RNA accumulation (prediction 6). For an intuitive illustration, consider that the levels of *CO* mRNA in the light are comparable or higher in *fkf1* mutants under long photoperiods compared to the wild-type under short photoperiods (compare Figure 3B with 3A), yet the cognate *FT* mRNA levels are much lower in the mutants (Figures 3C and 3D). Direct interaction of FKF1 with CO protein might enhance CO function (Fukamatsu et al., 2005), providing a mechanism based on known components. Light-stabilized CO protein would then activate *FT* transcription in an FKF1-dependent manner. This was achieved in a revised model by using the FKF1 protein profile to drive the transcription of *FT* in addition to its effect on *CO* (model 3F2, see Supplemental Data). This speculative model matched wild-type *FT* mRNA waveforms well and lacked the morning-specific peak of FT expression observed with earlier models (Figure 4). As *FKF1* is not expressed in the morning, simulated *FT* mRNA expression remains low at this time even if *CO* RNA is present. Thus the postulated function of *FKF1* provides a parsimonious molecular mechanism for the morning "gate" (prediction 1). Constitutive expression of *FKF1* and *GI* was insufficient to activate *FT* expression immediately after dawn (Sawa et al., 2007), however, indicating that further analysis of the morning "gate" is warranted. FKF1 was estimated to increase *FT* transcription 10-fold in our model compared to the *fkf1* mutant, highlighting the importance of FKF1 as a photoperiodic regulator (Imaizumi and Kay, 2006). FKF1 increased *CO* transcription by only 35%, suggesting that FKF1-independent factors are also important in regulating *CO* mRNA levels (Fornara et al., 2009; Imaizumi et al., 2005).

The relationship between the two effects of *FKF1*, on *CO* and *FT*, is unclear. A possible extension to model 3F2 was to propose that FKF1 functioned together with CO protein, and that this mechanism activated both *FT* and *CO* transcription (prediction 7). This parsimonious hypothesis predicted that CO protein would be required for the *FKF1*-dependent shoulder of *CO* mRNA under long photoperiods. New experimental data for the *CO* mRNA levels of seven *co* mutant alleles failed to support this notion (Figure S7). Our results therefore predict that *FKF1* functions differently to regulate *CO* and *FT*, indicating that a novel regulatory mechanism is involved in the control of *FT*. It is possible that this function is *GI*-dependent, as *GI* regulates flowering via the circadian expression of *CO* (as in our models) but also by a genetically-separable mechanism (Gould et al., 2006; Mizoguchi et al., 2005).

Tuning the Mechanisms of Day-Length Perception

The circadian clock models entrained stably to a limited range of exotic light-dark cycles with total durations that varied from 24 hr (Figure 5). The models were developed using data from only constant conditions and 12L:12D cycles (Locke et al., 2005a; Locke et al., 2005b), so more flexible models might be constructed based upon new data on the clock components under other conditions. The mathematical theory of coupled oscillators (Guckenheimer and Holmes, 1983) shows that stable entrainment occurs when the system parameters are within an area of parameter space described as the Arnold tongue. Changing the period of the entraining cycle significantly away from the period of the oscillator moves the system outside the Arnold tongue. Consistent with theory, the clock model then becomes quasi-periodic, as also reported for circadian rhythms in many species.

Using model simulations, we predicted that discrimination between long and short light intervals will be greatest when the total duration of the entraining cycle is 24 hr (Figure 5; prediction 8). This qualitative prediction is reminiscent of the classic experiments on soybean (Hamner and Takimoto, 1964) and matches more detailed data from the hamster, where photoperiodic regulation of the reproductive system also showed the greatest amplitude under 24 hr cycles (Elliott, 1974). This effect also can be related to the theory of coupled oscillators (Guckenheimer and Holmes, 1983). Altering the duration of the entraining cycle by a moderate amount relative to the period of the oscillator moves the system within the Arnold tongue, and alters the phases of the clock components. The circadian clock is presumed to be adapted to the 24 hr entraining cycles in which it has evolved: the clock components (and outputs such as *CO*) will be regulated with peak phases that are physiologically optimal (Pittendrigh and Daan, 1976). Changing the duration of the entraining cycle will alter their phases and lead to a suboptimal response, either slowing growth (Dodd et al., 2005), or in this case weakening the photoperiodic sensor.

Our models illustrate how the external coincidence mechanism has evolved in *Arabidopsis* to a more elaborate form than Bünning's original hypothesis. CO protein was the first molecular correlate of photoperiod to be identified and its dual regulation by the clock (transcriptionally) and by light (post-translationally) forms an external coincidence detector. The photoperiod-sensitive entrainment of the *CO* mRNA rhythm extends beyond the simplest external coincidence hypothesis and has potential functional significance in detecting photoperiod change, but may require specialized circadian timing, as discussed above. FKF1 forms a second external coincidence detector that regulates *CO* mRNA (Imaizumi et al., 2003) and we show that it is crucial in activating *FT* (prediction 6). As CO and FKF1 function together to regulate *FT* at the end of the photoperiod, separating their expression in time might in principle be sufficient to prevent flower induction under short photoperiods, as proposed by the internal coincidence hypothesis (Pittendrigh, 1960). It will be interesting to determine whether the entrainment of their circadian rhythms (and the rhythms of other clock-controlled

regulators) responds differently to photoperiods, as this would be required to introduce an aspect of internal coincidence (as implied in Imaizumi and Kay, 2006). In contrast, another photoperiodic mechanism appears to control the degradation rate of cry2 protein at the start of the day (El-Din El-Assal et al., 2001). The effect of cry2 degradation on photoperiodism is unclear, and our current models match *FT* mRNA profiles without this modulation of light input.

The existence of multiple photoperiod sensors has important consequences for understanding plant physiology. First, it greatly increases the potential for specialized photoperiodic sensors to control different physiological responses within a single species, including responses of vegetative organs. Second, it is unclear which (or how many) of the molecular mechanisms will be conserved across species with similar photoperiodic responses. Finally, it increases the possibility that species with different photoperiodic responses might differ radically in their molecular mechanisms. It is therefore all the more striking that homologs of *CO* and *FT* have been implicated in the short-day photoperiodic response of the dicots *Ipomoea nil* (Hayama et al., 2007) and poplar (Bohlenius et al., 2006) and of the monocot rice (the genes Hd1 and Hd3a, respectively, reviewed in Hayama and Coupland, 2004; Izawa, 2007). The profile of *Hd1* RNA strongly resembles that of *CO* (Figure S8a), such that Hayama et al. (Hayama et al., 2003) proposed that a single change of sign, equivalent to making *CO* a negative regulator of *FT*, would be sufficient to convert the long-day response of *Arabidopsis* to the short-day response of rice. Using RNA profiles of *Hd1* and *Hd3a* under long and short photoperiods, we constructed the simplest model of the rice photoperiod sensor based upon our *Arabidopsis* models (see supplemental data). The proposed repressive function of *Hd1* could fully account for the photoperiodic regulation of the mean level of *Hd3a*, because the coincidence of light with *Hd1* RNA in the evening changes significantly between long and short photoperiods. There is, however, little or no difference in *Hd3a* expression in the evening, so coincidence does not directly explain the temporal profile. Peak *Hd3a* expression occurs in the morning (Figure S8b), and matching this timing required a separate (and unknown) clock-regulated factor distinct from the *Hd1* repressor. It will be interesting to determine how the morning-specific photoperiodic regulator of *Hd3a* relates to the well-described, evening-specific regulators in *Arabidopsis*. There are now several possible candidates in rice (reviewed in Hayama and Coupland, 2004; Izawa, 2007). Recent data give this added relevance for *Arabidopsis*, because *FT* can also be expressed in the morning in some conditions (Corbesier et al., 2007), suggesting that the rice regulatory mechanism might also be present in *Arabidopsis*.

The photoperiodic switch is part of a broader network of developmental pathways and environmental responses that control the flowering of *Arabidopsis* (Boss et al., 2004). Models of this broad network (Welch et al., 2003) have the exciting potential to link our detailed molecular mechanisms to larger-scale phenological models. These already have widespread applications in crop scheduling and crop improvement (Adams et al., 2001; Hammer et al., 2006) and have been successfully applied to *Arabidopsis* development (Wilczek et al., 2009).

EXPERIMENTAL PROCEDURES

Plant Growth and RNA Assays

Seeds of *fkf1*, the *co* alleles and cognate wild-types were generously supplied by G. Coupland (Koeln) or by the Nottingham *Arabidopsis* Stock Centre. Plants were grown as described (Locke et al., 2005b) under 16L:8D for 10 days at 22°C. Samples were harvested 13h after lights-on; RNA was extracted and analyzed by qRT-PCR as described (Locke et al., 2005b).

Computational Methods

Quantitative expression profiles for *CO* and *FT* mRNA under various conditions were digitized from charts or graphs in the literature or kindly provided by the original authors (see Supplemental Data). Timeseries data were numbered (see Table S1), normalized and checked for consistency (see Supplemental Data). Twenty-eight timeseries from wild-type plants were used for most model training and validation, with ten further timeseries from *toc1* and *fkf1* mutants. Models were constructed as ordinary differential equations in Matlab (Mathworks, Cambridge UK); SBML versions will be available from the Biomodels repository upon publication (Le Novere et al., 2006), and in versions compatible with the Circadian Modeling simulation interface (available at www.amillar.org/Downloads.htm). Model equations and parameters are presented in the supplemental data. Parameters were estimated by fitting to the relevant data (for the wild-type, to the training data sets 1, 3, 8, and 9; see Table S1), using a boundary value solver to ensure that the model produced stable, limit cycle solutions (see supplemental data). Models 3F1 and 3F2 including FKF1 function are described as speculative, because there is much less quantitative timeseries data available for FKF1 in the literature than for *CO* and *FT*. We test these models only in 8L:16D and 16L:8D conditions, where FKF1 data are available.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, two tables, and eight figures and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)01487-1.

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